Nanotechnology Characterization Laboratory

NCL News

Accelerating the transition of concepts to clinical applications

June 2010

The NCL Turns 5

The National Cancer Institute (NCI) has a long history of support for biomedical nanotechnology research, funding hundreds of investigators through its Unconventional Innovations Program (UIP) and Alliance for Nanotechnology in Cancer. In 2004, the Alliance queried its investigators about what they saw as the greatest challenge in progressing their promising nanotech cancer drugs into clinics. The PIs answered that, in addition to funding, they needed access to instrumentation and expertise to meet FDA regulatory requirements to enter clinical trials. To get a drug into clinics, researchers have to show that the drug can be reproducibly manufactured and that it is efficacious and safe. For nanotech cancer drugs, this is necessarily an interdisciplinary venture — it requires the expertise of materials scientists, biologists, chemists, toxicologists, pharmacologists, immunologists, and others. The UIP investigators said they needed access to such expertise, and to instrumentation and techniques from outside their own disciplines.

The Nanotechnology Characterization Laboratory (NCL) was conceived in 2004 in response to this need. The NCL is a formal interagency collaboration among the National Cancer Institute (NCI), the National Institute of Standards and Technology (NIST), and the Food and Drug Administration (FDA) and is operated through the NCI's Federally Funded Research & Development Center (FFRDC) at SAIC/NCI-Frederick.

NCL began operations in 2005, when it hired its key scientists: Chemist, Dr. Anil Patri; Toxicologist, Dr. Stephan Stern; and Immunologist, Dr. Marina Dobrovolskaia. Their first item of business was to develop and validate protocols for the preclinical characterization of biomedical nanomaterials. These protocols would eventually become the foundation of NCL's Assay Cascade. The Assay Cascade serves as a standardized set of experiments for the physicochemical, *in vitro*, and *in vivo* study of biological nanomaterials, and as a public resource (the protocols are available for download from the NCL website). The NCL Assay Cascade was designed with input from NIST and the FDA to ensure it is applicable to a wide variety of nanomaterials and that it is tailored to regulatory requirements. Currently there are more than 20 protocols available for download; the majority of these are now being evaluated as formal Standards by ASTM and ISO.

In 2006, the NCL began accepting nanomaterials for characterization. Candidates for NCL characterization are selected through an application process (more details can be found at http://ncl.cancer.gov). NCL characterization is free to the client. NCL scientists work with clients to determine the best course of action, i.e., which protocols best address gaps in their developmental "critical path". Depending on the developmental stage, complete characterization of a nanoparticle may take up to a year (or more!). Upon completion, a fully inclusive document, which captures the results of all NCL experiments, will be drafted and presented to the client. These Client Reports, generally more than 100 pages of scientific data, are intended to support investigational new drug (IND) or investigational device exemption (IDE) applications with the FDA.

In that first fully operational year, with a staff of just four scientists and three technicians, the NCL developed six collaborations and generated four client reports. At the culmination of 2009, NCL was operating with a staff of more than 20 scientists and technicians, including chemists, biologists, dedicated electron microscopy technicians, dedicated histology technicians, and a statistician, and generated eight client reports. NCL's list of collaborators has grown to over 65, and includes laboratories from academia, small biotech companies,

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large pharmaceutical companies, and US government institutions.

NCL is now a well-established laboratory with state-of-the-art instrumentation: five electron microscopes, an imaging mass spectrometer, a three Tesla clinical MRI, and a wide range of other spectroscopy, light scattering and chromatography instruments suitable for thorough chemical and biological characterization of nanomaterials. In addition, the NCL's *in vivo* animal studies now include the potential for tests in non-human primates through collaboration with the FDA's National Center for Toxicological Research (NCTR).

Through five years of operation, NCL's highest priority has remained the same: helping to advance its collaborators' nanotechnologies to the clinic. The characterization data generated by the NCL has aided a handful of investigators in successful IND or IDE applications with the FDA and helped many advance their material towards clinical trials and/or generate venture capital.

Structure-activity relationship (SAR) studies are an important part of the NCL's charter and a contribution to the cancer and nanotech research communities. These studies help to reveal trends observed in NCL studies, and help to shape future nanomaterial development. NCL also has made contributions to the Environment, Health and Safety field with SAR studies of the penetration of nanoscale titanium dioxide particles in rodents, pig skin, and human skin, SAR studies of macrophage uptake of gold nanoparticles, and studies of the relationship between nanomaterials and autophagy (a type of cellular regulation). These findings are disseminated to the public through publication in peer-reviewed journals, and presentations at scientific conferences and workshops. ■

New ICH Guidelines

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)1 was established in 1990 with the goal of harmonizing the regulatory requirements for pharmaceuticals among the United States, Europe, and Japan. The objective of the ICH is to implement quality, safety, and efficacy guidelines for pharmaceutical development, such that expensive and time-consuming research need not be duplicated for international release of needed medications. FDA experts on harmonization and international relations act as liaisons to the ICH, and the FDA often uses the ICH guidance documents to inform US regulatory guidance.

The majority of investigations done by the NCL are ultimately intended for inclusion in Investigational New Drug (IND) and Investigational Device Exemption (IDE) filings with the FDA; therefore, the NCL makes every attempt to stay abreast the most current guidelines for the FDA approval process. Any recommendations made by the ICH are of direct importance to the NCL and its clients wishing to pursue further advancement of their nanomaterial with regulatory agencies.

The most recent issuance of "Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals" [M3(R2)] by the ICH,[2] offers recommendations aimed at expediting progress to clinical trials and reducing the extent to which human, animal and other resources are consumed. This guidance details that preclinical studies should be performed to assess toxicity concerns before beginning human clinical trials, and should include general and reproductive toxicity studies, toxicokinetic and pharmacokinetic studies, and pharmacology studies.

Historically, acute toxicity results from single or multiple administrations over a 24 hour period, but are now often substituted by short-duration exposures to a particular substance.[3] Generally, all toxicology and pharmacokinetic studies geared towards IND filings are required to be performed under Good Laboratory Practice (GLP) conditions. GLP experiments, which ensure high quality research data, are costly and time consuming as a result of documentation and procedural requirements.

This recent M3(R2) document makes the recommendation that non-GLP dose-escalation or dose-range finding studies can be used in place of GLP acute toxicity studies. These non-GLP studies must be administered via the same route as the intended clinical route of administration, and this clinical administration must also have corroborating GLP repeat-dose toxicity studies. This recommendation was approved by the European Union in June 2009 and will become effective in December 2009. The Ministry of Health, Labour and Welfare of Japan or the United States FDA have yet to approve this, but are soliciting comments.

The NCL performs acute toxicity and repeat-dose studies in rodents as a routine component of nanomaterial characterization. An evaluation of nanomaterial acute toxicity is an important part of the *in vivo* tier of the NCL's Assay Cascade. The NCL is not a GLP certified facility. However, this

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recommendation by the ICH, if finalized, will allow for use of NCL-generated toxicology data in rodents as part of IND filings with the FDA. This will be advantageous to NCL's collaborators, allowing them to include NCL data in their regulatory filings, without the added cost and time of repeating acute toxicity studies under GLP conditions. ■

[1] The ICH website: http://www.ich.org

[2] The ICH Nonclinical Safety Guidance M3(R2) report can be found at: http://www.ich.org/LOB/media/MEDIA5544.pdf

[3] http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079270.pdf

NCL Connections

NCTR Collaboration

The NCL has now established a collaboration with the National Center for Toxicological Research (NCTR) to facilitate nanotechnology studies in large animals (non-human primates). NCTR is the FDA's internationally recognized research center. Its mission is to develop science-based best-practice standards, guidance, and tools and to incorporate toxicological advancements into the regulatory process. NCTR is located in Jefferson, Arkansas, and has state-of-the-art facilities and expertise for conducting toxicological studies in a variety of animal species.

NCTR and NCL have several common goals -- NCTR and NCL share an interest in understanding mechanisms of nanoparticle toxicity, and exploring nanoparticle biological behavior including biodistribution, pharmacokinetics, and clearance. Both NCTR and NCL also have common goals in understanding how nanoparticle platforms may mediate certain adverse drug effects in humans, such as interaction with the immune system and alteration of hemodynamics, which cannot be appropriately evaluated with in vitro or rodent models.

NCL is partnering with NCTR in a way that leverages both NCTR and NCL core expertise and resources to facilitate nanotechnology studies. NCL will provide NCTR with physicochemical characterization of nanomaterials to elucidate any environmental, health, and safety concerns, while the NCTR will provide NCL with the use of Rhesus Macaques at the NCTR facility. The goal of these studies is furtherance of a framework for effective risk identification, assessment, and regulatory evaluation of nanotech products. NCL and NCTR plan to perform these non-human primate studies for GLP-quality pharmacokinetic studies, in support of NCL-collaborator IND applications, and to provide primate data for interspecies pharmacokinetic comparisons. Measures of toxicity, specifically immunotoxicity, that are amenable to blood analysis will also be included.

NCL and NCTR estimate that we will conduct three such animal studies per year or one every four months, allowing time for animal recovery and clearance of the administered drugs. Most of the nanoparticles tested by the NCL have clearance half-lives on the order of 24 hours or less, so this schedule should provide ample time for drug clearance between studies. NCL and NCTR both expect these collaborative studies will facilitate development and regulatory review of new nanotech clinical products and aid in the development of nanotechnology-based platforms and tools for medical research and clinical applications. ■

More information about NCTR can be found at:

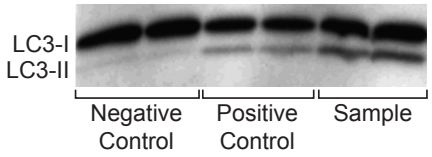
http://www.fda.gov/AboutFDA/CentersOffices/NCTR/default.htm

NCL Protocol: Autophagic Dysfunction

Autophagy is a lysosomal process through which damaged organelles and long lived proteins are recycled. Autophagy is also considered a potential pathway of programmed cell death, often referred to as Type II programmed cell death (apoptosis is Type I programmed cell death). There are many examples of nanoparticle-induced autophagic dysfunction (AD) available in the literature,[1] and recent evidence that AD may be a relevant mechanism of nanoparticle toxicity *in vivo*.[2]

Although the implications of autophagy-nanomaterial interaction are not completely understood, AD is considered a marker of cell stress.[3] Additionally, there is concern that AD itself may result in injury or exacerbation of disease. Thus, AD is an important pathway to monitor when investigating new nanomaterial platforms. To help further the understanding of autophagy-nanomaterial interaction, the NCL has developed several methods to screen for nanoparticle-autophagy interaction.

Common methods used to detect autophagic dysfunction include direct morphological assessment via electron microscopy, in both cell culture and tissue samples, as well as use of lysosomal dyes and protein modification assays. The NCL currently has two protocols optimized for detection of autophagic dysfunction. NCL method GTA-11 measures the autophagy biomarker LC3-II, and GTA-12 measures uptake of the lysosomal dye, Lysotracker Red DND-99 (Invitrogen[4]). Full experimental details for both protocols can be found at http://ncl.cancer.gov/NCL_Method_GTA-11.pdf and http://ncl.cancer.gov/NCL_Method_GTA-12.pdf



GTA-11 specifically measures lipidation and cleavage of MAP LC3-I to LC3-II by immunoblotting. LC3-I is a cytosolic protein that, during initiation of autophagy, is conjugated to phosphotidylethanolamine to the produce LC3-II. LC3-II is a component of the autophagosomal membrane, and thus the amount of LC3-II subunit expression can be used as a surrogate marker of autophagy.

The alternate autophagic dysfunction assay, GTA-12, utilizes Lysotracker Red DND-9, a cationic fluorescent dye that preferentially accumulates into the acidic lysosomal or autolysosomal compartment. Therefore, the amount of dye taken up by cells in culture can be used as an indicator of lysosome content. Decreases in dye uptake, relative to control, indicate conditions such as decreased lysosomal stability, or, conversely, increases in dye uptake are indicative of autophagic dysfunction.

A variety of other conditions, apart from autophagic dysfunction, could potentially cause either changes in lysosomal dye uptake or LC3-II expression. With any positive result in this assay, treatment-related changes should be further evaluated by morphological assessment, using techniques such as electron microscopy, to confirm autophagosome involvement. ■

[1] *Biochem Biophys Res Commun* 2005; 337: 52-60. *Nano Lett* 2006; 6:2826-2832. *Circ J* 2006; 70:129-140. *Am J Physiol Cell Physiol* 2006; 290: 1495-1502. *Eur J Pharmacol* 2007; 568:89-98.

[2] *J Mol Cell Biol* 2009; 1:37-45.

[3] *Aquat Toxicol* 2007;84(1):80-91. *Erratum in: Aquat Toxicol* 2008;86(3):457.

[4] The NCL does not imply endorsement or recommendation of any of the above mentioned suppliers of commercial materials. Their inclusion is for informational purposes only; equivalent supplies from alternate vendors can be substituted.

FAQs

Q: Does every nanoparticle submitted to the NCL undergo every experiment in the full NCL Assay Cascade? Who/what determines which assays will be used to test my nanoparticle?

A: Not every submitted nanoparticle goes through the entire assay cascade. Every particle does go through "the prescreen"–physicochemical tests to confirm formulation identity, and tests to verify the formulation is free of bacteria, yeast, mold, or endotoxin contamination. Beyond the prescreen, the collaborator and NCL scientists jointly determine and prioritize characterization experiments. Factors influencing this plan include the type of nanomaterial (e.g. many of the physicochemical assays are material-specific), the collaborator's previous characterization of the material, the collaborator's desired knowledge of the material, and the intended final application of the material.

Q: How many nanoparticles may I submit for characterization?

A: Generally speaking, the NCL would prefer to characterize only one lead nanomaterial -- your most promising candidate formulation for promotion to clinical applications. In addition, the NCL usually requires control nanomaterials and synthetic precursors for experimental comparison.

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If you've not yet decided on a lead formulation, NCL may be able to help with lead selection, but these experiments have a lower priority at NCL than IND–directed characterization experiments. NCL has over 50 collaborations, and we prioritize experiments on a weekly basis based on our characterization demands and results (efficacy, pharmaceutical viability, etc.). We assign a higher priority to formulations that have already gone through the lead selection process.

That said, if something unexpected happens and you decide you're not taking that formulation to clinic, you are welcome to apply for characterization of other formulations, and NCL is happy to apply whatever it learned about the first particle to subsequent formulations. Previous acceptance of one formulation, however, does not automatically guarantee acceptance of a subsequent formulation for NCL characterization.

Q: How long does NCL nanoparticle characterization take?

A: The entire assay cascade can be completed in one to one-and-a-half years. However, many factors may shorten or lengthen this timeframe. Issues with nanomaterial sterility, endotoxin levels, and production of adequate amounts of material can delay the process.

Here is a general timeline for NCL characterization:

1-1.5 months

In general, we perform the "NCL prescreen" within 45 days of receiving your nanomaterial. The prescreen consists of tests for contamination (bacterial, yeast, mold and endotoxin), as well as preliminary physicochemical tests for size distribution, such as dynamic light scattering (DLS).

1-3 months

Once the prescreen is complete, if everything looks good, we continue with physicochemical and *in vitro* characterization. This includes measurement of physicochemical characteristics like drug loading, charge, purity, stability and surface characteristics and evaluation of *in vitro* blood contact properties (plasma protein binding, hemolysis, platelet aggregation, complement activation, etc.). This phase of characterization also includes evaluation of effects on cell viability using a variety of methods. This "second phase" of NCL characterization generally takes about 45-60 days. This phase can be slowed considerably if one of our experiments appears to give spurious results (results not in-line with the results of the other experiments) or if we discover a property of the nanomaterial that was unanticipated (e.g. unexpected toxicity or interference with the assay).

3-12 months

Physicochemical and *in vitro* characterization continues during this period, and, finally, if everything continues to look promising, we begin *in vivo* experiments. We usually start with a dose-range finding toxicity study to determine the toxic dose and target-organs for any toxic response to the nanomaterials. This takes 1-2 months. This is usually followed by a biodistribution study, using available methods to track the distribution and clearance of the nanomaterial, and/or an efficacy study, evaluating the nanomaterial in either xenograft, transgenic, or orthotopic cancer models in rodents. Each of these studies typically takes 2-3 months to plan, conduct, and analyze the results. Finally, we usually conduct the full subacute toxicity study as the final phase of NCL characterization. This study is very involved and may take 4-6 months to complete. It is also the most expensive, and likely the most important for an IND.

Keep in mind that the above timeline assumes everything goes well and that there are no unexpected results – this is uncommon, since there are almost always unexpected results!

Q: How will I access NCL data?

A: NCL data is provided to our collaborators via webconference throughout the study period, several times a year, with ample opportunity for discussion of results and development of upcoming experimental plans. When characterization is complete, a fully inclusive report is presented to the collaborator detailing results of all NCL experiments.

Q: Can I publish data generated by NCL?

A: Certainly. NCL scientists greatly appreciate the opportunity to collaborate on publications. NCL data are intended to be used in regulatory filings, in publications, and to garner interest from venture capital. ■

Recent NCL Publications:

Ambiguities in applying traditional Limulus Amoebocyte Lysate tests to quantify endotoxin in nanoparticle formulations.
Dobrovolskaia M, Neun BW, Clogston JD, Ding H, Ljubimova J, McNeil SE.
Nanomedicine, 2010 5(4), to be published in June.

Translational considerations for cancer nanomedicine.
Stern ST, Hall JB, Yu LL, Wood LJ, Paciotti GF, Tamarkin L, Long SE, McNeil SE.
Journal of Controlled Release. Published online April 10, 2010.

Lack of significant dermal penetration of titanium dioxide (TiO_2) from sunscreen formulations containing nano- and sub-micron-size TiO_2 particles.
Sadrieh N, Wokovich AM, Gopee NV, Zheng J, Haines D, Parmiter D, Siitonen PH, Cozart CR, Patri A, McNeil SE, Howard, PC, Doub WH, Buhse LF.
Toxicological Sciences, Published online Feb 15, 2010.

Minireview: Nanoparticles and the Immune System.
Zolnik BS, González-Fernández A, Sadrieh N, Dobrovolskaia MA.
Endocrinology, 2010; 151(2): 458-465.

Nanomaterial standards for efficacy and toxicity assessment.
Adiseshaiah P, Hall JB, McNeil SE.
Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology 2009; 2(1):99-112.

Evaluation of nanoparticle immunotoxicity.
Dobrovolskaia MA, Germolec DR, Weaver JL.
Nature Nanotechnology 2009; 4(7):411-414.

Nanoparticle interaction with plasma proteins as it relates to particle biodistribution, biocompatibility and therapeutic efficacy.
Aggarwal P, Hall JB, McLeland CB, Dobrovolskaia MA, McNeil SE.
Advanced Drug Delivery Reviews 2009; 61(6):428-437.

Interaction of colloidal gold nanoparticles with human blood: effects on particle size and analysis of plasma protein binding profiles.
Dobrovolskaia MA, Patri AK, Zheng J, Clogston JD, Ayub N, Aggarwal P, Neun BW, Hall JB, McNeil SE.
Nanomedicine 2009; 5(2):106-117. ■

Upcoming Conferences

Canadian Society of Pharmaceutical Sciences
Location: Vancouver, British Columbia
Date: June 2 – 5, 2010
Web site: http://www.cspscanada.org/symposium_2010.aspx

5th Annual Greener Nanoscience Conference
Location: Portland, OR
Date: June 16 – 18, 2010
Web site: http://oregonstate.edu/conferences/event/greenernano2009/index.htm

2nd International Symposium on Biological Applications of Dendrimers
Location: Porquerolles, France
Date: June 23 – 26, 2010
Web site: http://www.cinam.univ-mrs.fr/biodend2010/index.html

37th Annual Meeting and Exposition of the Controlled Release Society
Location: Portland, OR
Date: July 10 – 14, 2010
Web site: http://www.controlledrelease.org/meeting/default.cfm?CFID=4517233&CFTOKEN=29305479

American Chemical Society Fall 2010 National Meeting
Location: Boston, MA
Date: August 22 – 26, 2010
Web site: http://www.acs.org/meetings

Materials Research Society, Functionalized Nanobiomaterials for Medical Applications
Location: Denver, CO
Date: October 4 – 7, 2010
Web site: http://www.mrs.org/s_mrs/sec.asp?CID=25035&DID=290998

2010 Materials Research Fall Meeting
Location: Boston, MA
Date: November 29 – December 3, 2010
Web site: http://www.mrs.org/s_mrs/sec.asp?CID=16777&DID=216967

Pacifichem 2010
Location: Honolulu, HI
Date: December 15 – 20, 2010
Web site: www.pacifichem.org ■

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